APPENDIX A

The Estimation of the Relative Dimer Equilibrium Constants for Insulin-Insulin, Insulin-Lispro and Insulin-Aspart

In order to evaluate the relative equilibrium constants for dimerization shown in Equation 1, we used circular dichroism spectroscopy.

Equation 1.

$I + A \rightarrow IA \quad K = [IA]/[I][A]$ where I is insulin, A is analog (lispro or aspart) and [I] means the concentration of insulin in moles/liter.

Literature values [1] for the dimerization constant of human insulin vary widely over the range of 0.09 to 1.4×10^5 depending on the analytical technique used and the formulation conditions. We have evaluated the relative dimerization constants using the following scheme.

1. Formulation – Insulin and Insulin analogs (U 100 manufacturer's primary packaging) were diluted with insulin diluent (glycerin, phosphate buffer, pH 7.4) without added preservatives or excess zinc. Circular dichroism spectra were obtained using an Olis spectrometer calibrated with camphorsulfonic acid.
2. Samples of insulin and the analog were made at stock concentrations such that mixing would yield a final concentration of 0.04 mg/ml, 0.125 mg/ml and 0.5 mg/ml.

Figure 1, attached shows the CD spectrum of a solution of insulin that is mostly monomeric. The spectrum shows a sharp peak at 225 nm and no hint of a shoulder.

[1] Hassiepen and colleagues, Eur. J. Biochem., 1998, p. 580.

Figure 2, shows a spectrum of 0.5 mg/ml insulin solution where the solution is about 35% monomer, 58% hexamer and the balance monomer. The peak has shifted to 212 nm and a significant shoulder is apparent at 222 nm.

Figure 3 shows a spectrum of 0.125 mg/ml insulin solution that is predominantly dimeric (>65%) with 30% monomer and the balance hexamer. The peak and shoulder are more pronounced.

The calculations for percent monomer, dimer and hexamer were performed using an Excel spreadsheet and the values of dimerization of 1.4×10^5 and hexamerization as 4×10^8 as in reference 1. To evaluate the effect of an analog on the insulin we made solutions of insulin at 0.06 mg/ml and analog at the same concentration so that the total insulin plus analog concentration was 0.12. We used the ellipticity of the shoulder at 222 nm to evaluate the amount of dimer formed and compared it to the amount of dimer formed with insulin. The data are shown in Table 1 below.

Insulin Concentration	Lispro Concentration	Aspart Concentration	Percent of Dimer Formed
0.125	0	0	100% (defined)
0.06	0.06	0	44%
0	0.125	0	16%
0.06	0	0.06	48%
0	0	0.125	19%

Conclusions: From this simple study we have shown that the insulin-analog heterodimer equilibrium binding constant is larger than the analog-analog homodimer but considerably less than the binding strength of the insulin homodimer.

Figure 1 -- Insulin Circular Dichroism
Concentration = 0.04 mg/ml
Predominately Monomer

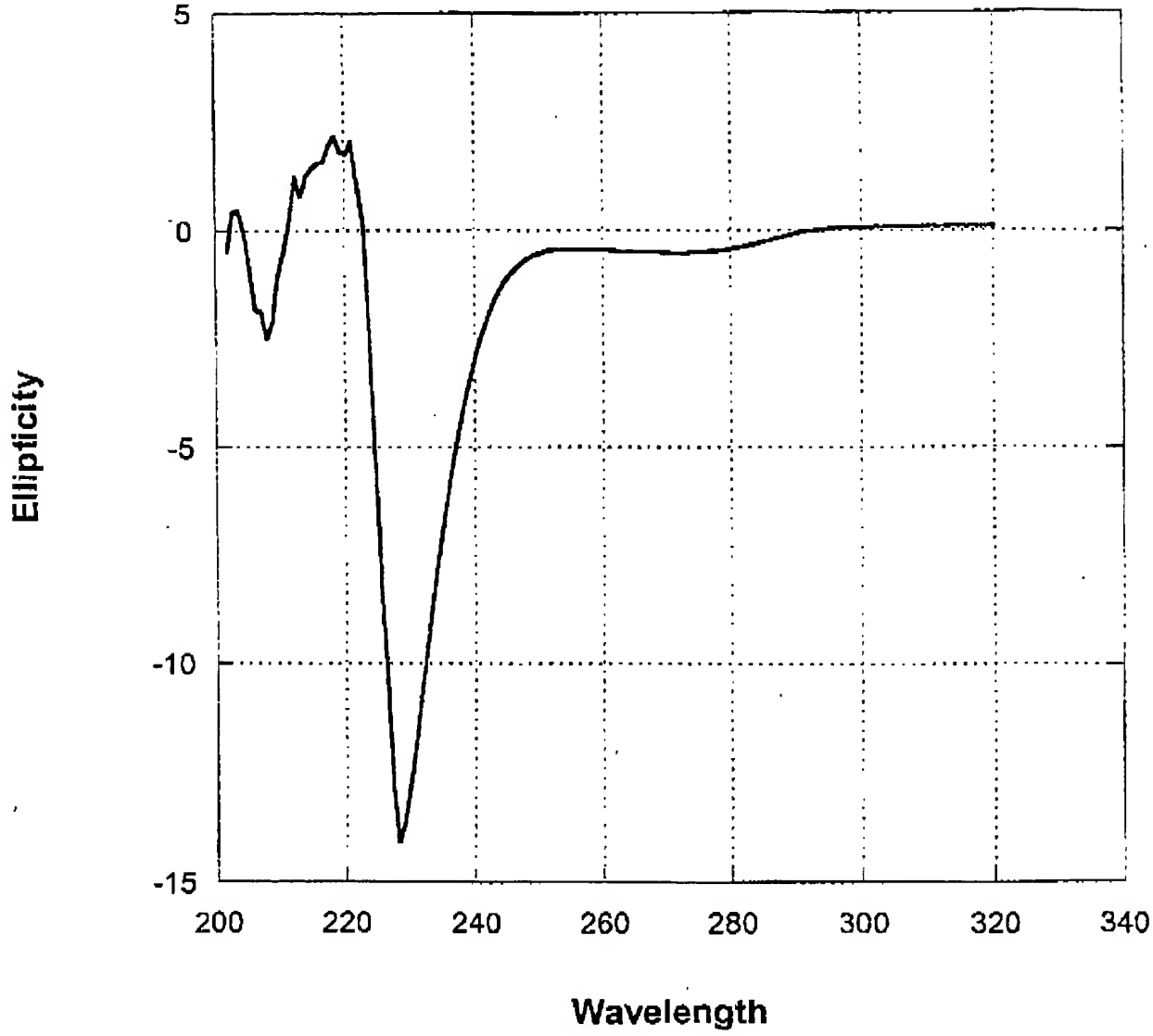

Figure 2, Insulin Circular Dichroism
Concentration = 0.5 mg/ml
Approximately 35% Dimer

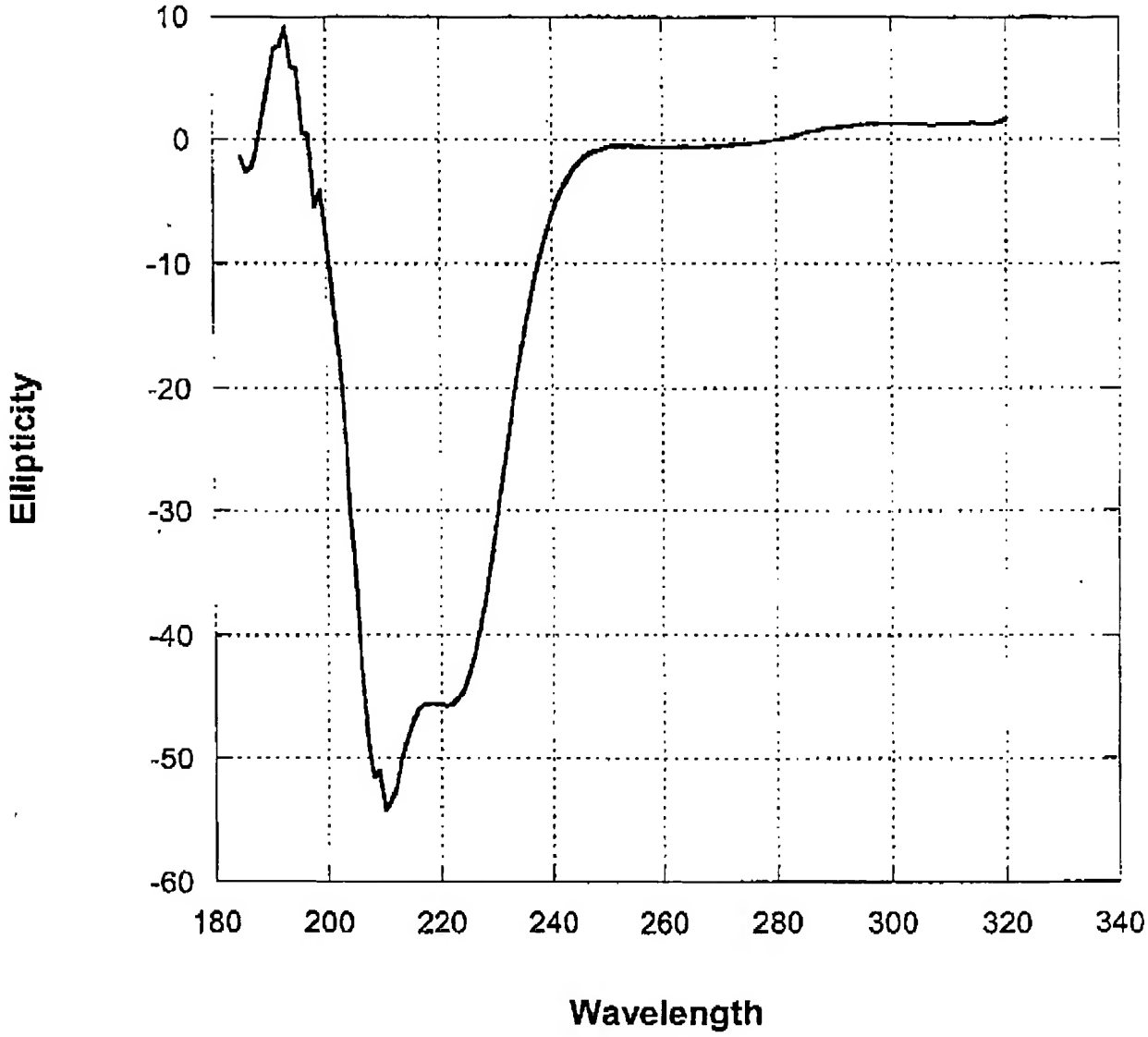

Figure 3, Insulin Circular Dichroism
Concentration = 0.125 mg/ml
Dimer Concentration approximately 65%